HYBRID SPECIATION IN WILD SUNFLOWERS[1]

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ABSTRACT

Hybrid speciation refers to the establishment of novel hybrid genotypes that are reproductively isolated from their parental species and genetically stabilized. Most frequently, reproductive isolation is achieved via an increase in ploidy. However, in some instances new hybrid species arise and become reproductively isolated without a change in chromosome number, a process known as diploid or "homoploid" hybrid speciation. The annual sunflowers of the genus *Helianthus* provide a well-studied example of this latter mode of speciation. Here, I review this work, placing individual studies in their proper context. These include (1) computer simulations that describe the evolutionary conditions under which hybrid speciation is most likely; (2) molecular phylogenetic studies that document the origins of three hybrid sunflower species; (3) comparative genetic mapping studies that describe the karyotypic changes associated with hybrid speciation; (4) experimental re-creations of homoploid hybrid species that allow genotypic and phenotypic comparisons between synthetic and ancient hybrid lineages; (5) quantitative trait locus (QTL) studies that describe the genetic basis of phenotypic differences between the parental species and the mode of gene action underlying the generation of extreme phenotypes in hybrids; (6) phylogeographic studies that estimate the ages and number of origins of each hybrid species; (7) selection studies that measure the strength of selection on individual traits and QTLs in synthetic hybrids transplanted into hybrid habitats; and (8) candidate gene studies that search for correlations between candidate genes for ecological divergence and traits and QTLs shown to be under selection in the habitats of the hybrid species. Ongoing work includes searches for the molecular signature of selection during hybrid speciation, surveys of gene expression shifts associated with hybrid speciation, and experiments that evaluate the role of new hybrid gene combinations versus reproductive isolation in the ecological divergence of hybrid lineages.

Key words: ecological divergence, genetic mapping, *Helianthus*, homoploid hybrid speciation, QTLs, reproductive isolation.

Species often come into contact before reproductive barriers are fully formed, resulting in the formation of hybrid swarms or hybrid zones. The consequences of hybridization are most frequently discussed in relation to the reproductive isolation of the hybridizing lineages. That is, the hybrid zones may be stable, reflecting a balance between dispersal and selection (Barton & Hewitt, 1985). Alternatively, they may be ephemeral, due either to the strengthening of reproductive barriers and cessation of hybridization (Noor, 1999) or to the weakening of reproductive barriers and fusion of the hybridizing lineages (Wolf et al., 2001). All of these consequences are well documented empirically, although their frequencies remain poorly understood.

The consequences of hybridization may also be discussed with respect to adaptive evolution (Anderson, 1949; Arnold, 1997). In stable hybrid zones, for example, favorable alleles will move rapidly across hybrid zones, and thereby contribute to adaptation in the recipient species. This "adaptive trait introgression" may be common, but it is difficult to detect because the introgressing alleles will spread rapidly to fixation and are unlikely to be "caught in the act" (Barton, 2001). Less frequently, hybrids may escape from hybrid zones, which allows fit hybrid gene combinations to become established (Buerkle et al., 2000). Although seemingly rare, these stabilized hybrid lineages are much easier to detect and study. As a consequence, much of what we know about the role of hybridization in adaptive evolution comes from the study of these hybrid races or species (Rieseberg et al., 2003).

Finally, a significant component of the literature on hybridization and its consequences has focused on the means by which these new hybrid lineages become reproductively isolated from their parental species. Most commonly, isolation arises as a byproduct of hybrid genome duplication or allopolyploidy (Stebbins, 1950). However, reproductive isolation sometimes develops without a change in chromosome number, a mode of speciation called diploid or homoploid hybrid speciation (Grant, 1981).

In this paper, I review a series of studies conducted by my lab over the past 15 years that describe the origins of three diploid hybrid sunflower species, the means by which they became reproductively isolated from their parental species, and the theory underlying the process. This narrative serves several purposes. First, our theoretical and empirical work provide

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convincing evidence that hybridization can contribute to adaptive evolution and suggest that it may sometimes serve as a mechanism for large and rapid evolutionary transitions. Second, the work illustrates the kinds of experiments required to rigorously assess hybridization's role in evolution. Finally, the sunflower hybrid speciation story is currently scattered across the literature, appearing in more than 25 papers. This narrative ties these pieces together, placing individual studies in their proper context.

HYBRID SPECIATION: THEORY

Hybrid speciation refers to the process in which novel genotypes generated by hybridization become isolated from their parental species and other hybrid genotypes and become genetically stabilized. Reproductive isolation is straightforward for hybrids with increased ploidy because hybrids between polyploids and their diploid parents typically have an odd number of genomes and may therefore be largely sterile (Grant, 1981). In contrast, homoploid hybrid speciation represents a challenge to evolutionary theory, because homoploid hybrid lineages must become established despite the possibility of backcrossing with their parental species (Turelli et al., 2001).

Three mechanisms have been proposed by which a homoploid hybrid may become reproductively isolated from its parental species. First, the new hybrid lineage may diverge karyotypically from its parental species through the sorting of chromosomal rearrangements that differentiate the parental species (Stebbins, 1957; Grant, 1981), and/or by the establishment of new chromosomal rearrangements induced by recombination (Rieseberg et al., 1995b). Second, it has been speculated that hybrid founder events may facilitate hybrid speciation by providing initial spatial isolation for the new hybrid lineage (Charlesworth, 1995). Third, a hybrid lineage may colonize a new habitat or niche and thus become ecologically isolated from its parental species (Grant, 1981). Hybrid species often occupy habitats that are very different from those occupied by their parental species (Rieseberg, 1997; Wang et al., 2001; Gross & Rieseberg, 2005). This is probably because a novel hybrid lineage is more likely to survive if it experiences little competition with its parental species and if divergence in habitat preference results in some spatial isolation with the parental species (Templeton, 1981; Charlesworth, 1995).

Three simulation studies have examined the process of homoploid hybrid speciation (McCarthy et al., 1995; Buerkle et al., 2000, 2003). The first of these was a spatially explicit individual-based simulation, which monitored the consequence of hybridization between species that differed in karyotype (McCarthy et al., 1995). There were two possible outcomes—the generation of a stable hybrid zone or the origin of a novel hybrid genotype that displaced the parental species. The latter outcome was considered hybrid speciation, but it probably is more correctly viewed as the merger of species through hybridization. In most documented hybrid species, the parental species continue to coexist with the derived hybrid (Rieseberg, 1997). Thus, our group developed a computer simulation model to investigate the conditions for this more conventional kind of hybrid speciation (Buerkle et al., 2000), which is the focus of this paper. Our model was similar to that of McCarthy et al. (1995) in its inclusion of chromosomal rearrangements, but differed by providing a novel habitat in which hybrids were favored, as well as the possibility of spatial separation between the novel hybrid habitat and that of the parental species.

Three possible outcomes were possible in our model: hybrid zone stasis, hybrid speciation, and adaptive trait introgression, in which one of the parental species colonizes the novel habitat by acquiring advantageous alleles from the other parent. This latter outcome was by far the most common. Hybrid speciation was less frequent, but it did occur at a significant rate when the sterility barrier isolating the parental species was weak and hybrid genotypes were strongly favored in novel, open habitat (i.e., strong ecological selection). In contrast, hybrid zone stasis was rare, apparently because of the presence of novel habitat in which hybrid genotypes had a fitness advantage.

The conditions required for the independent evolution of the new hybrid species following its origin are surprisingly different from those favoring establishment in the first place (Buerkle et al., 2000). Although a weak sterility barrier between the parental species favors speciation, it also translates into an even weaker barrier between the new hybrid lineage and its parents. As a consequence, the genetic integrity of the new hybrid lineage cannot be maintained in parapatry with parental populations. Thus, for hybrid lineages to evolve independently from parapatric parental populations, the initial sterility barrier between the parental species must be strong, a requirement that greatly reduces the likelihood of this mode of speciation. Alternatively, isolation of the hybrid neospecies can be maintained by strong ecological selection and/or spatial isolation, a result that accords well with earlier verbal predictions that hybrid speciation is most likely following hybrid founder events, in which a few hybrids found a new population that is ecologically and spatially isolated from the parental species (Charlesworth, 1995; Rieseberg, 1997).

The third simulation study (Buerkle et al., 2003) was an extension of Buerkle et al. (2000), except that the novel habitat for hybrids was eliminated, the sizes of the hybridizing parental populations were allowed to vary, and the extent of spatial isolation was reduced. This new model allowed us to assess the relative frequency of the four most important outcomes of hybridization: hybrid zone stasis, hybrid speciation, adaptive trait introgression, and extinction of one of the parental species (typically the rare one). Hybrid zone stasis was most common, occurring in 84% of simulations. Extinction of the rarer species occurred only when the sterility barrier was weak and was essentially always the result of adaptive trait introgression. Hybrid speciation occurred in only 2.1% of the simulations and was favored by a weak sterility barrier between the parental species and moderate to strong ecological selection. However, hybrid speciation was almost always accompanied by extinction of one of the parental species. These results indicate that the presence of novel habitat greatly facilitates hybrid speciation, as has been suggested by numerous students of hybrid speciation over the past century (e.g., Kerner, 1894–1895; Grant, 1981; Templeton, 1981; Arnold, 1997; Rieseberg, 1997). Also, without an open niche, one of the parental species is replaced by the hybrid, and total species diversity remains unchanged.

All three simulations studies have one remarkable result in common. When hybrid speciation does happen, it occurs quickly, often in fewer than fifty generations. Thus, McCarthy et al. (1995) referred to homoploid hybrid speciation as a punctuated mode of speciation, in which long periods of hybrid zone stasis are followed by abrupt transitions in which a new fit hybrid genotype becomes established.

HYBRIDIZATION AND THE EVOLUTIONARY HISTORY OF SUNFLOWERS

The role of hybridization in the diversification of North American sunflowers (*Helianthus* L.) was initially explored by Charles Heiser and his students (Heiser, 1947, 1949, 1951a, b; Heiser et al., 1969). Heiser documented the occurrence of natural hybrids between the widespread, common sunflower, *H. annuus* L., and several of its congeners, including *H. argophyllus* Torr. & A. Gray (Heiser, 1951a), *H. bolanderi* A. Gray (Heiser, 1949), *H. debilis* Nutt. (Heiser, 1951b), and *H. petiolaris* Nutt. (Heiser, 1947). This hybridization, Heiser argued, allowed *H. annuus* to acquire favorable alleles from the locally adapted species, thereby increasing its ecological amplitude and facilitating range expansion. In some instances, hybridization was believed to have contributed to the formation of introgressive races (*H. annuus* subsp. *texanus* Heiser in Texas (Heiser, 1951b) and *H. bolanderi* subsp. *bolanderi* in California (Heiser, 1949)) or new species (*H. neglectus* Heiser (Heiser et al., 1969)).

My laboratory re-examined these hypotheses using a combination of molecular marker surveys and molecular phylogenetic analyses (Dorado et al., 1992; Rieseberg et al., 1988, 1990a, b, 1991a, b; Rieseberg, 1991). We verified much of Heiser's work, including the occurrence of natural hybridization between *Helianthus annuus* and each of the species listed above (Dorado et al., 1992; Rieseberg et al., 1988, 1990a, b, 1998), as well as the introgressive origin of *H. annuus* subsp. *texanus* (Rieseberg et al., 1990b). On the other hand, the molecular evidence failed to support a hybrid origin for *H. neglectus* or *H. bolanderi* subsp. *bolanderi* (Rieseberg et al., 1988). Thus, while the morphological criteria employed by Heiser accurately identified contemporary hybrid swarms or zones, their utility in the detection of ancient, stabilized hybrid lineages was limited. This was not unexpected, as it has long been recognized that morphological intermediacy may sometimes result from processes other than hybridization (Gottlieb, 1972).

In addition to verifying or refuting putative examples of hybrid lineage formation, molecular phylogenetic analyses detected unsuspected hybrid origins of three species (Fig. 1): *Helianthus anomalus* Blake, *H. deserticola* Heiser, and *H. paradoxus* Heiser (Rieseberg et al., 1990b, 1991a; Rieseberg, 1991). The three hybrids appear to be derived from the same parental species, *H. annuus* and *H. petiolaris*. However, there are subtle differences in the parental chloroplast and nuclear ribosomal DNA haplotypes found in each hybrid, suggesting that each was independently derived (Rieseberg, 1991; Rieseberg et al., 1991a). Over the past decade we have exploited this replicated, natural hybrid speciation experiment, in combination with theoretical studies, to derive a more predictive theory of hybrid speciation. This body of work is described below.

NATURAL HISTORY

The three hybrid species and their parents are self-incompatible, insect-pollinated annuals, with the same chromosome number (n = 17). All five species are native to the continental United States. The two parental species have widespread and broadly overlapping distributions that are centered in the U.S. Great Plains (Fig. 2). They differ in soil preferences,

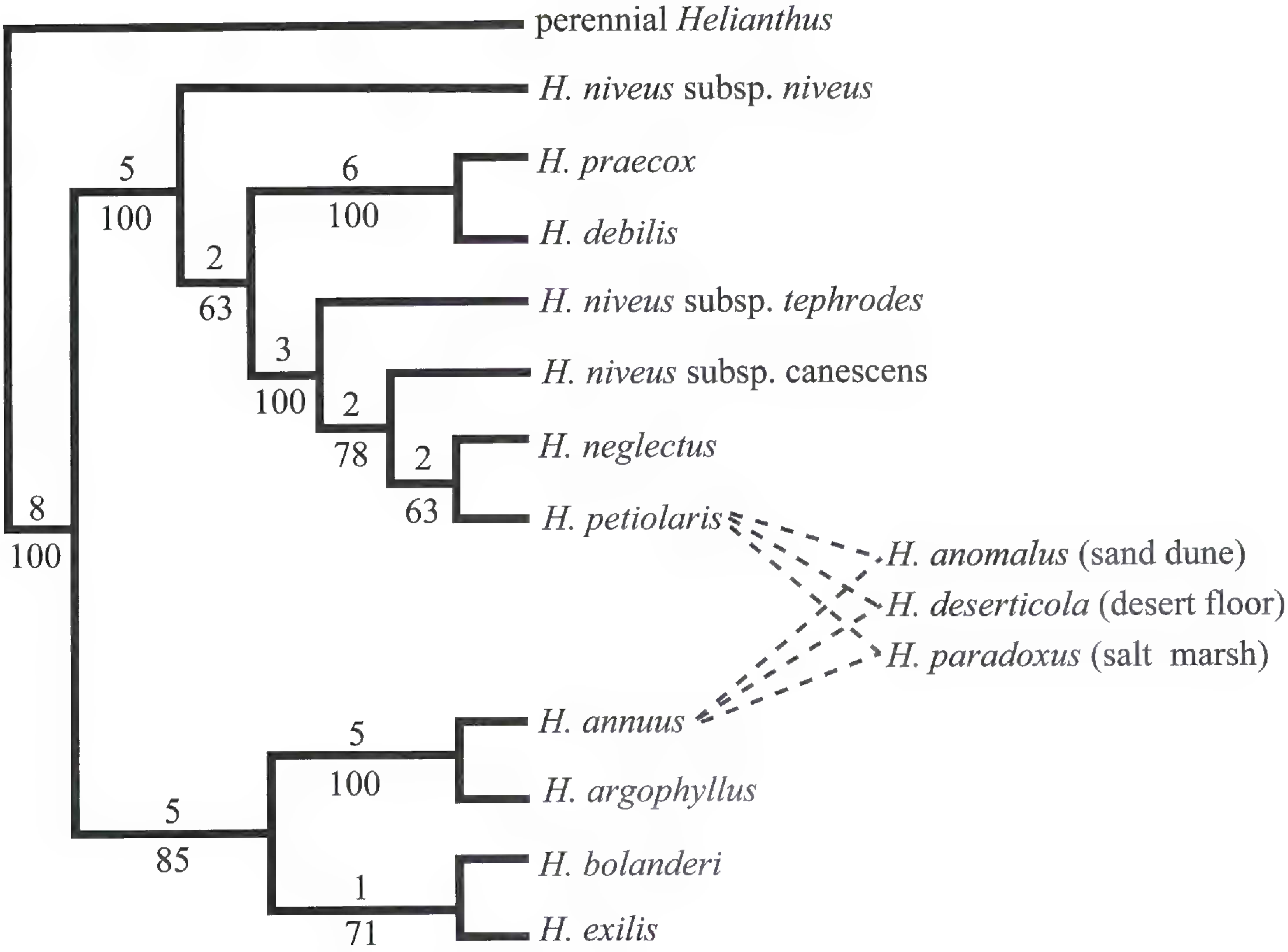
perennial *Helianthus*
H. niveus subsp. *niveus*
H. praecox
H. debilis
H. niveus subsp. *tephrodes*
H. niveus subsp. canescens
H. neglectus
H. petiolaris
H. anomalus (sand dune)
H. deserticola (desert floor)
H. paradoxus (salt marsh)
H. annuus
H. argophyllus
H. bolanderi
H. exilis
5
100
6
100
2
63
3
100
2
78
2
63
8
100
5
100
5
85
1
71

Figure 1. Phylogenetic tree for *Helianthus* sect. *Helianthus* based on combined chloroplast DNA and nuclear ribosomal DNA data (redrawn from Rieseberg, 1991, fig. 9). The number of mutations are given above and bootstrap percentages below each branch. Dashed lines indicate parentage of homoploid hybrid species.

however, with *Helianthus annuus* largely restricted to heavy, clay soils, and *H. petiolaris* to dry, sandy soils (Fig. 3). Nonetheless, these two habitats are often found in close proximity in the central and western United States, resulting in the production of numerous hybrid swarms and hybrid zones. The hybrid zones are narrow, often less than 30 m, and little evidence of introgression is found outside of the hybrid zones, apparently due to the synergistic action of several reproductive barriers (Rieseberg et al., 1995a, 1999b; Schwarzbach et al., 2001).

The three hybrid species are much more limited in geographic distribution than their parents (Fig. 2), with restriction of *Helianthus deserticola* to the Great Basin Desert in Nevada, Utah, and northern Arizona, *H. anomalus* to a handful of sand dune habitats in Utah and northern Arizona, and *H. paradoxus* to saline wetlands in western Texas and New Mexico (Fig. 3). Despite proximity to parental populations, no natural hybrids have been reported between the three ancient hybrid species and their parents. In contrast, the three hybrid species are almost completely allopatric to each other, but I have observed hybrids between *H. anomalus* and *H. deserticola* at the only site where they co-occur, Little Sahara Recreation Area in Central Utah.

Wild sunflower species produce an indehiscent one-seeded fruit called an achene (or cypsela). Although the achenes are eaten by small mammals and birds, dispersal by way of the animal feces seems unlikely because most seeds are likely to be digested. It seems more likely that sunflowers are dispersed by large mammals such as bison. Wild sunflower achenes are covered with small hairs that can bind to fur, and sunflower achenes have been reported in buffalo fur (Asch, 1993). There also are reports from early settlers of an association between bison and sunflowers. For example, in 1839 journalist Matthew Field described how "among the sunflower beds the huge back of a buffalo here and there was seen, as the ponderous brute broke down the stalks before him while pressing toward a fresher pasture ground" (cited in Asch, 1993: 12).

In addition to their role in dispersal, the disturbance generated by bison may have created opportunities for hybridization. Hybridization has long been known to be associated with natural and anthropogenic disturbance (Anderson, 1948), and, as shown by

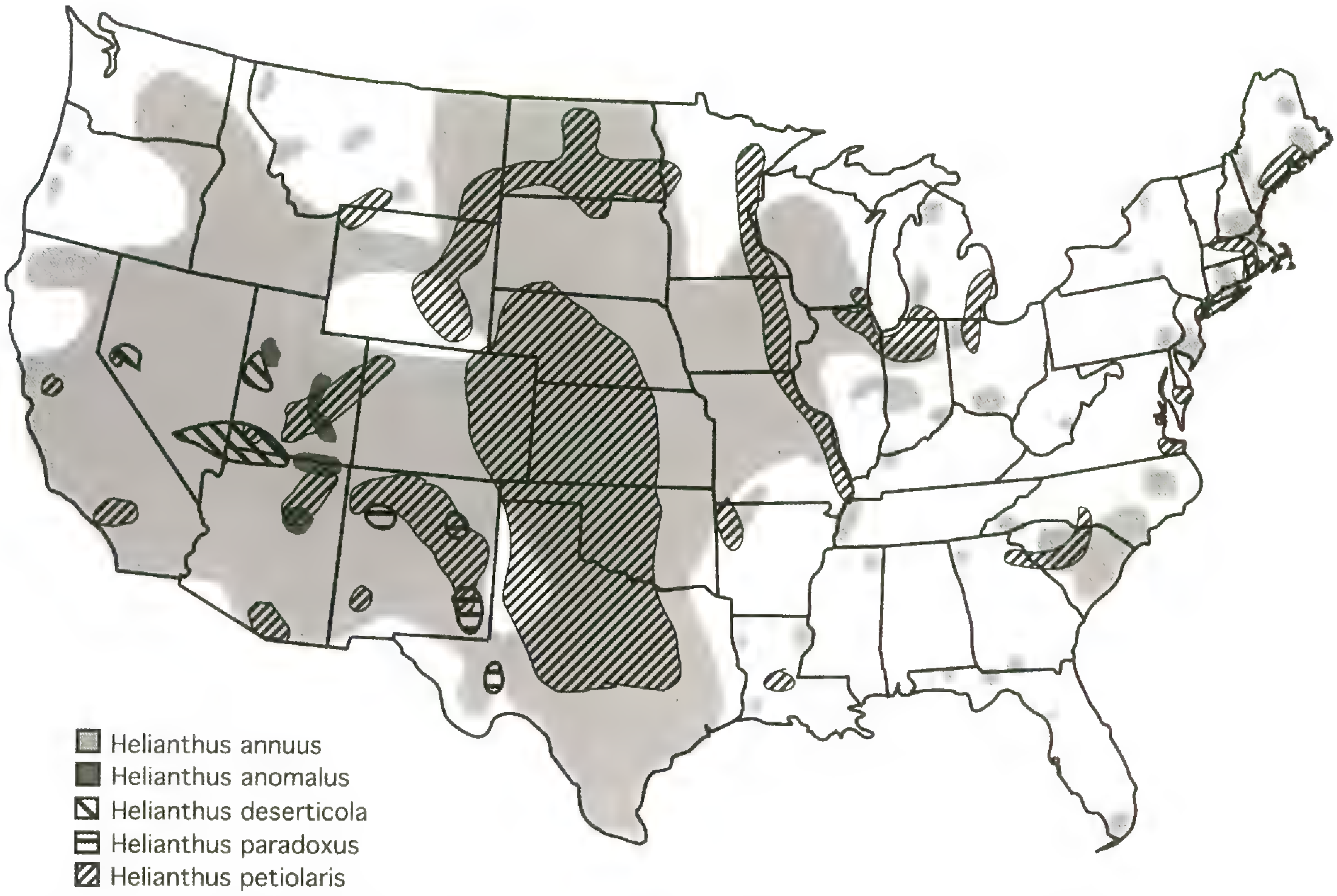

Figure 2. Geographic distributions of the two parental species and their three hybrid derivative species.

the following passage from Barsness (1985: 1), the impact of bison on the Great Plains was profound: "The [bison] herds marked their territory with trails, thousands of them, some shallow traces, some eight-to-ten-inch trenches, others so deep that the animal's sides would rub the embankments." Reflecting either cause or coincidence, microsatellite divergence places the origin of the three hybrid *Helianthus* species between 63,000 and 210,000 generations ago (Schwarzbach & Rieseberg, 2002; Welch & Rieseberg, 2002b; Gross et al., 2003), or just after the colonization of North America by bison ca. 200,000 years ago (Dary, 1974).

Karyotypic Evolution

As discussed earlier, theory indicates that the development of reproductive isolation between the new hybrid lineages and their parental species may be facilitated by rapid karyotypic evolution (Grant, 1981; McCarthy et al., 1995; Buerkle et al., 2000, 2003). Most verbal and simulation models have assumed that the karyotypic change occurs through the sorting of preexisting chromosomal rearrangements that differentiate the parental species (Stebbins, 1957; Grant, 1958). However, Templeton (1981) noted that recombination in hybrids might lead to additional chromosomal breakage.

To assess the role of genomic restructuring in hybrid speciation, we have generated detailed genetic linkage maps for the three hybrid species, *Helianthus anomalus*, *H. deserticola*, and *H. paradoxus*, as well as their putative parental species, *H. annuus* and *H. petiolaris* (Rieseberg et al., 1993, 1995b; Ungerer et al., 1998; Burke et al., 2002; Rieseberg et al., 2003). These maps were developed using a combination of AFLP, RAPD, and SSR (microsatellite) markers, with more than 650 markers placed on each map. Alignment of orthologous markers across the five maps allowed us to reconstruct the chromosomal rearrangements that have accompanied hybrid speciation.

All three hybrids have undergone massive karyotypic re-organization. Not only does each of the hybrid species possess a unique combination of parental chromosomal rearrangements, but also a minimum of three, two, and five chromosomal breakages (and an equal number of fusions) are required to achieve the *Helianthus anomalus*, *H. deserticola*, and *H. paradoxus* genomes, respectively (Rieseberg et al., 1995b, 2003; Burke et al., 2004; Lai et al., 2005). We have previously shown that approximately 50% of the reproductive barrier between *Helianthus* species is caused by chromosomal rearrangements (Rieseberg et al., 1999b). Furthermore, crossability and interfertility studies (Rieseberg, 2000, unpublished) indicate the

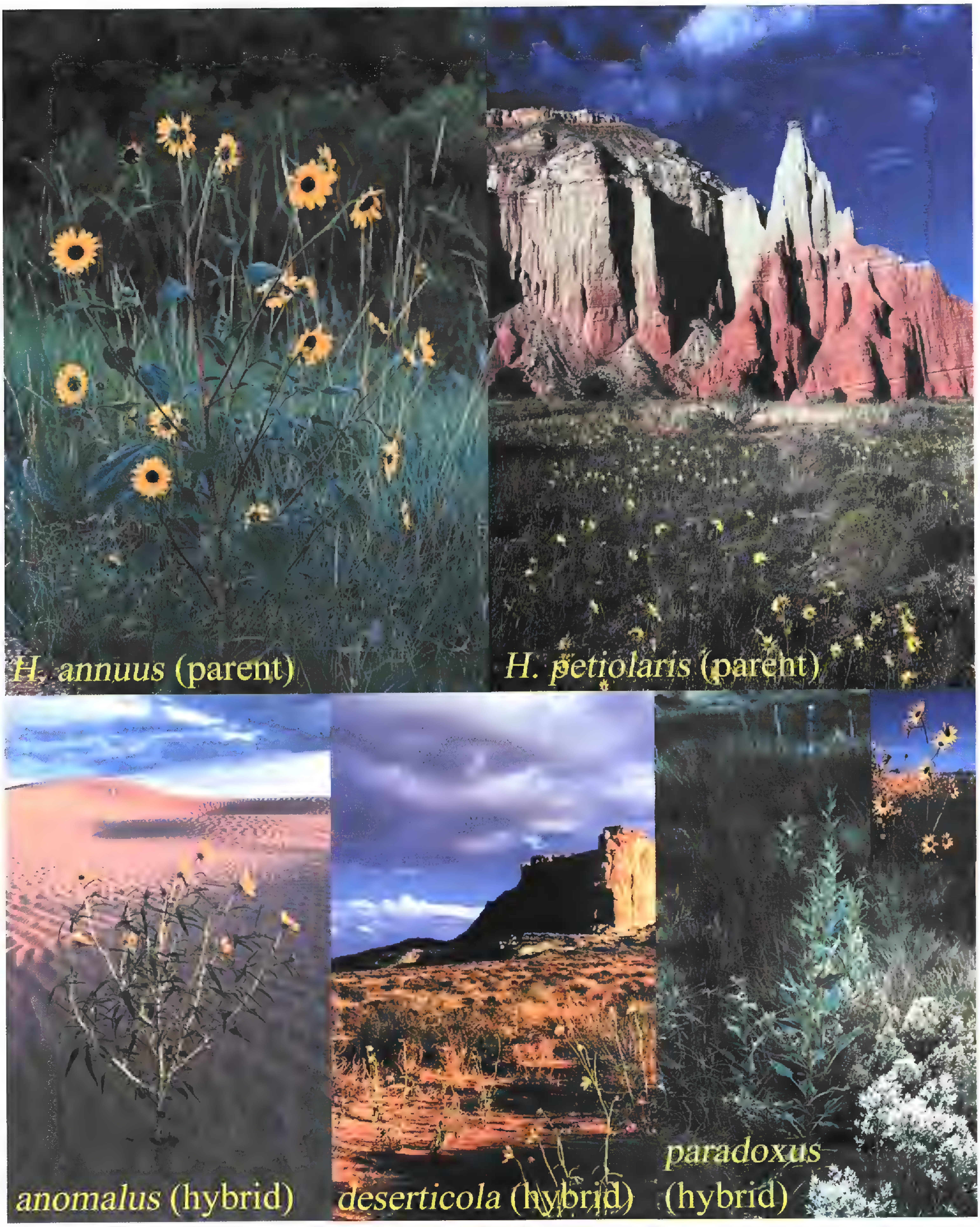

Figure 3. Photographs of the two parental species and their three hybrid derivative species in typical habitats. Photographs by Jason Rick.

three hybrid species are strongly isolated from each other and from their parental species (Fig. 4). Thus, as predicted by theory, rapid karyotypic evolution in the hybrid sunflower species appears to have facilitated the development of reproductive isolation with their parental species (Templeton, 1981; Buerkle et al., 2000).

Experimental Syntheses of Homoploid Hybrid Species

There is a rich literature describing the recovery of fertility in synthetic hybrid lineages and the degree of their isolation from their parental species (Stebbins, 1957; Grant, 1966a, b, 1981). These early studies were important because they demonstrated that

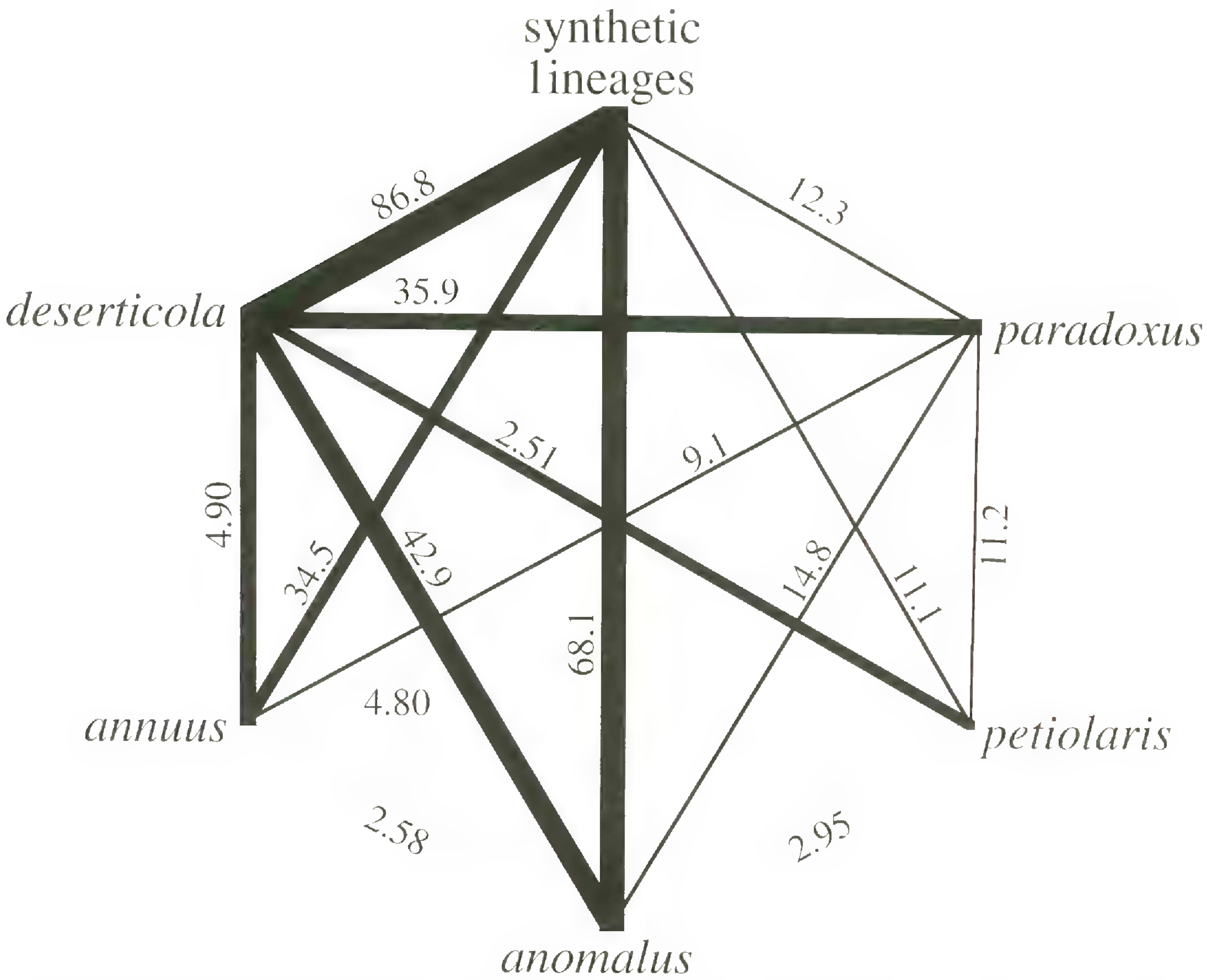

Figure 4. Crossability and interfertility among the two parental species, synthetic hybrid lineages, and three hybrid derivative species. Percentages indicate pollen viability of first generation hybrids. Line thickness is proportional to reproductive compatibility.

homoploid hybrid speciation was feasible. However, they did not attempt to replicate naturally occurring hybrid species or compare the newly synthesized hybrid lineages with those already existing in nature. The *Helianthus* work fills this important gap in the literature.

We attempted to replicate the origins of the hybrid species by synthesizing hybrids between the two parental species, *Helianthus annuus* and *H. petiolaris*, and subjecting the synthetic hybrids to selection for fertility over several generations (Rieseberg et al., 1996). Because sunflowers are self-incompatible and early generation hybrids are semi-sterile, hybrid speciation in this group likely involved both backcrosses and filial crosses. Therefore, we employed both kinds of crosses, but varied the order of backcross and filial generations in three independent experiments.

The results were gratifying. After only four generations of selection, pollen fertility in the three hybrid lineages had increased from 5.6 to 91.8%, indicating that the recovery of fertility not only was feasible, but that it potentially could occur very rapidly (Ungerer et al., 1998). In addition, the synthetic hybrid lineages were strongly isolated from their parental species, with mean pollen fertility ranging from 29 to 42% for crosses with *Helianthus annuus* and 10 to 13% for crosses with *H. petiolaris* (Fig. 4; Rieseberg, 2000). Remarkably, the three independently generated hybrid lineages were highly interfertile when crossed with each other; mean pollen fertility ranging from 83 to 89% (Rieseberg, 2000). This result implies that the three hybrid lineages have converged onto a fairly similar set of parental genes or chromosomal segments. Indeed, meiotic analyses indicated the synthetic lineages were similar in karyotype, varying in only one or two reciprocal translocations (Rieseberg, 2000).

To explore the genomic similarity of the synthetic hybrid lineages, we screened ca. 60 individuals from each lineage with 197 molecular markers specific to one or the other parental species (Rieseberg et al., 1996). Genomic congruence, as measured by the ϕ coefficient (a standard measure of association that can vary from −1 to 1; Sokal & Rohlf, 1995), ranged from 0.65 to 0.75 ($P < 0.0001$) for comparisons among the three hybrid lineages, a result that accords well with the high fertility reported for crosses between these lineages (Rieseberg, 2000).

Given the congruence in genomic composition among the synthetic hybrid lineages, it seemed sensible to determine if the three natural hybrid species had found a similar solution to the problem of hybrid sterility. This was a more difficult problem that required surveying natural populations of both *Helianthus annuus* and *H. petiolaris* to determine the likely parental origin of each marker mapped in the three hybrid species (Rieseberg et al., 1993, 1995b, 2003; Ungerer et al., 1998). In all, 427, 290, and 325 of the markers mapped in *H. anomalus*, *H. deserticola*, and *H. paradoxus*, respectively, could be assigned to one or the other parental species and thus were informative with respect to genomic composition (Rieseberg et al., 2003). Genomic congruence was high for comparisons between the three synthetic hybrid lineages and either *H. anomalus* or *H. deserticola*: ϕ ranged from 0.44 to 0.50 ($P < 0.0001$) for comparisons with *H. anomalus* (Rieseberg, 2000) and 0.53 to 0.59 ($P < 0.0001$) for comparisons with *H. deserticola* (Rieseberg, unpublished). In contrast, little congruence was observed between the three synthetic lineages and *H. paradoxus*: 0.28 to 0.34 ($P < 0.01$). Also, note that levels of genomic congruence accord closely with crossing relationships (Fig. 4); the synthetic hybrids are most compatible and most interfertile with *H. anomalus* and *H. deserticola* as predicted by genomic congruence, but least compatible and least interfertile with *H. paradoxus*. From a broader perspective, these results indicate that fertility selection plays a major role in determining hybrid genomic composition and that the number of solutions to the problem of hybrid sterility may be fairly limited. Clearly, *H. paradoxus* has found a different solution than the other taxa. Possibly, there were different proportions of the parental species in the hybrid zone from which *H. paradoxus* was founded and/or the genomic composition of *H. paradoxus* was strongly influenced by ecological selection (Lexer et al., 2003a, b; Rieseberg et al., 2003).

TEMPO OF HYBRID SPECIATION

Both theoretical and experimental studies of homoploid hybrid speciation indicate that it is likely to occur very rapidly (Stebbins, 1957; Grant, 1966a, b; Rieseberg et al., 1996; McCarthy et al., 1995; Buerkle et al., 2000), but until recently no methods have been available to test this prediction. We have devised a method for estimating the tempo of hybrid speciation in nature (Ungerer et al., 1998). The rationale for our method is that in a newly forming hybrid species the sizes of parental chromosomal segments are expected to become progressively smaller over time due to recombination (Fisher, 1953; Baird, 1995). However, continued reduction in segment size will be countered by stabilization of the hybrid species' genome; subsequent recombination will be among segments derived from the same parental species (Ungerer et al., 1998). This analysis represents an empirical application of R. A. Fisher's junctions approach (Fisher, 1953), which tracks parental species segments by monitoring recombination breakpoints or "junctions" between heterogeneous regions rather than all points on a genome. In a neutral case, we can imagine a "junctions clock," which is analogous to the molecular clock, but slows over time as heterozygosity decreases due to drift. By comparing the frequency spectrum of observed parental species' chromosomal segments with predictions based on computer simulations (Ungerer et al., 1998), it is possible to estimate the number of generations required to stabilize the genome of a hybrid species. Note that we are estimating the speed of hybrid speciation, not the age of the hybrid species.

To date, we have only estimated the tempo of speciation for one of the hybrid species, *Helianthus anomalus* (Ungerer et al., 1998). This species has the largest number of mapped species-specific markers, thereby allowing the most precise empirical estimates of chromosomal segment lengths (Ungerer et al., 1998). It seems likely that the hybrid species will arise in hybrid founder populations that are spatially and/or ecologically isolated from the parental species. Therefore, we modeled this scenario by simulating a hybrid swarm, starting with equal numbers of *H. annuus* and *H. petiolaris* individuals, and allowing recombination to take its course in small, closed populations of different sizes and under different selection regimes. The speed of hybrid speciation was estimated by comparing the sizes of chromosomal segments in the simulation studies with those of *H. anomalus*. Chromosomal segment sizes in the *H. anomalus* genome were large, suggesting that its genome became stabilized in 10 to 60 generations. This result accords well with earlier simulation studies of hybrid speciation in which new hybrid species typically became established in ca. 50 generations (McCarthy et al., 1995; Buerkle et al., 2000) and argues that the tempo of homoploid hybrid speciation, like allopolyploidy, is very rapid.

PHENOTYPIC DIVERGENCE

The three hybrid species inhabit what are arguably the most extreme habits of any sunflower (dune, desert

Table 1. Hypothetical example of transgressive segregation due to the complementary action of genes with additive effects (from Rieseberg et al., 2003).

	Phenotypic values			
QTLs	Species A (AA genotype)	Species B (BB genotype)	Transgressive F_2	Transgressive F_2
1	+1	−1	+1(AA)	−1(BB)
2	+1	−1	+1(AA)	−1(BB)
3	+1	−1	+1(AA)	−1(BB)
4	−1	+1	+1(BB)	−1(AA)
5	−1	+1	+1(BB)	−1(AA)
Total	+1	−1	+5	−5

floor, and salt marsh habitats; Fig. 3) and have diverged significantly from the parental species for many traits associated with these habitats (Schwarzbach et al., 2001; Rosenthal et al., 2002). An important question is whether hybridization could provide the phenotypic variation necessary for colonization of these habitats.

We addressed this question by comparing phenotypic variation in the three ancient hybrid species, synthetic hybrids (a BC_2 population of *Helianthus annuus* × *H. petiolaris*), and both parental species grown in a common greenhouse environment (Rosenthal et al., 2002; Rieseberg et al., 2003). A total of 40 morphological, life history, and ecophysiological traits were measured.

As expected, given their divergent habitat preferences, the ancient hybrid species differed significantly from the parental species for many of the measured traits: 20 for *Helianthus anomalus* (8 intermediate, 12 extreme), 14 for *H. deserticola* (4 intermediate, 10 extreme), and 24 for *H. paradoxus* (8 intermediate, 16 extreme). Intermediate traits are easily accounted for by hybridization because the entire phenotypic continuum between the parental species is found in segregating hybrids (Rieseberg et al., 2003).

Hybridization could account for most of the extreme traits as well. Indeed, for 28 of the 40 traits, BC_2 plants had phenotypic values that significantly exceeded those of the parental species, a phenomenon known as transgressive segregation (Grant, 1975). Overall, 10 of 12 (83.3%), 10 of 10 (100%), and 11 of 16 (68.8%) extreme traits in *Helianthus anomalus*, *H. deserticola*, and *H. paradoxus*, respectively, were within the range of the BC_2 population. The handful of trait values exceeding those in the BC_2 population might have been transgressive in a reciprocal backcross population or have arisen through mutational divergence rather than hybridization. In either case, it is clear that the majority of extreme traits observed in the ancient hybrid species could have been generated through hybridization. These results have now been replicated by similar common garden experiments conducted in the natural habitats of each of the parental species (Lexer et al., 2003a; Gross et al., 2004; Ludwig et al., 2004). Thus, transgressive segregation, which is commonly observed in segregating hybrid populations (Rieseberg et al., 1999a), provides a simple mechanism for the ecological divergence of hybrid lineages.

GENETICS OF TRANSGRESSIVE SEGREGATION

So how are extreme phenotypes generated in hybrid populations? Numerous possibilities have been suggested (deVicente & Tanksley, 1993; Rieseberg et al., 1999a), including an elevated mutation rate, reduced developmental stability, non-additive effects of alleles at the same (overdominance) or different (epistasis) loci, unmasking of rare recessive alleles, and the combination of alleles with effects in the same direction from both parental species (complementary gene action; Table 1).

We employed quantitative trait locus (QTL) methods to determine the mode of gene action underlying transgressive segregation in *Helianthus* (Rieseberg et al., 2003). Briefly, the same BC_2 population employed for phenotyping in the previous section was genotyped for 96 molecular markers, and 185 QTLs were detected for the 40 traits. Remarkably, 39% of the QTLs had effects in the opposite direction of species differences and 34 of 40 traits had at least one opposing QTL. That is, for these QTLs, the *H. annuus* allele produced a more *H. petiolaris*-like phenotype, whereas the *H. petiolaris* allele produced a phenotype that was in the direction of *H. annuus*. This is exactly the kind of genetic architecture that is conducive for transgressive segregation under the complementary gene action model. Hybrid individuals that combine all of the "plus" or all of the "minus" QTLs from both parents for a given trait will have transgressive phenotypes (Table 1). Although complementary gene action explained most of the transgressive phenotypes, epistatic interactions were detected for 18 traits as well. These results are similar to those obtained for cultivated plants; complementary gene action is most frequent, but epistasis and overdominance do sometimes contribute (deVicente & Tanksley, 1993; Rieseberg et al., 1999a).

GENETIC CORRELATIONS

Transgressive segregation via complementary gene action provides a means by which extreme phenotypes may be generated for individual traits. For a hybrid lineage to successfully colonize a new habitat, however, all of the trait differences must be combined into a single individual or genotype, which may be difficult because of linkage and/or pleiotropy for QTLs underlying key traits. Thus, genetic correlations may limit ecological divergence through hybridization.

To determine the role of genetic correlations in the ecological divergence of the hybrid sunflower species, we asked whether closely linked or pleiotropic QTLs in the BC_2 population described above have effects that are in the same direction with respect to the hybrid species phenotype (Rieseberg et al., 2003). These positive correlations would greatly facilitate ecological divergence and hybrid speciation.

We found closely linked QTLs were indeed positively correlated in direction of effect (Rieseberg et al., 2003: Fig. 1). The ϕ coefficient of association ranged from 0.32 ($N = 256$; $P < 0.001$) for *Helianthus paradoxus*, to 0.47 ($N = 256$; $P < 0.001$) for *H. anomalus*, to 0.53 ($N = 256$; $P < 0.001$) for *H. deserticola*. Thus genetic correlations likely facilitated rather than impeded the origins of the three species, particularly for *H. anomalus* and *H. deserticola*. However, our results also imply that genetic correlations may limit the number of ecologically relevant multi-trait phenotypes, perhaps explaining why only three new hybrid species have originated from this cross.

HYBRID SPECIES' GENOMIC COMPOSITION AND PREDICTED PHENOTYPES

Although studies of synthetic hybrids demonstrate that most of the phenotypic differences associated with each of the hybrid species could have arisen through hybridization, they fail to prove that this is what actually happened. It could be, for example, that the differences arose as a consequence of mutational divergence and that hybridization was incidental to phenotypic evolution. To distinguish between these two hypotheses, we compared the genomic composition of the three ancient hybrid species with predictions from the QTL analyses of the BC_2 population of *Helianthus annuus* × *H. petiolaris* (Rieseberg et al., 2003). That is, we asked whether the hybrid species had the predicted set of QTL alleles for producing their phenotypes. If hybridization played a key role in phenotypic divergence, then a significant correlation should be found between predicted and actual genomic composition.

Genomic composition of the ancient hybrid species did accord closely with predictions from the QTL analyses (Rieseberg et al., 2003: Fig. 2). The ϕ coefficient of association ranges from 0.56 ($N = 150$; $P < 0.001$) for *Helianthus paradoxus*, to 0.58 ($N = 193$; $P < 0.001$) for *H. anomalus*, to 0.65 ($N = 94$; $P < 0.001$) for *H. deserticola* (Rieseberg et al., 2003). Thus, hybridization does appear to be largely responsible for the phenotypic divergence of the three hybrid species.

Earlier in this paper, I reviewed evidence suggesting that fertility selection plays a major role in shaping hybrid genomic composition (Rieseberg et al., 1996), whereas the QTL comparisons described above suggest that phenotypic (presumably ecological) selection must be important as well (Rieseberg et al., 2003). What is the relative importance of these two modes of selection? A preliminary comparison of the two data sets indicates that in many instances there is concordance between the predicted genomic composition from fertility selection and the QTL analysis of phenotypic differences, perhaps implying overlap between hybrid incompatibility genes and those responsible for phenotypic differences. Also, the combined data set more completely accounts for hybrid genomic composition than either individual data set does alone (Rieseberg, unpublished).

PHYLOGEOGRAPHY

Comparisons of the genomic composition of the ancient hybrid species with synthetic hybrid lineages and predictions from QTL studies (above) suggest that homoploid hybrid speciation may be repeatable. Thus, we asked whether the ancient hybrid species had arisen multiple times in nature. This was accomplished by surveying natural populations of the parental species and their three hybrid derivatives for variation at chloroplast DNA and nuclear microsatellite loci (Schwarzbach & Rieseberg, 2002; Welch & Rieseberg, 2002b; Gross et al., 2003). We also assessed crossability and interfertility among populations of *Helianthus anomalus* and *H. deserticola*. In *H. anomalus*, the crossing studies were complemented by meiotic analyses of inter-populational hybrids to determine the chromosomal basis of variation in fertility.

For a single origin, all populations of a given hybrid species are expected to have the same chloroplast

DNA haplotype, to form a monophyletic lineage based on nuclear microsatellite loci, and to be highly interfertile (i.e., have a single karyotype) when crossed. For multiple origins, different populations of a species may vary in chloroplast DNA haplotype and the species is unlikely to be monophyletic with respect to nuclear microsatellite loci. Also, significant variation in fertility due to karyotypic variability is predicted for inter-populational crosses. Note that multiple chloroplast DNA haplotypes and polyphyly for nuclear microsatellite loci can also result from a single origin followed by introgression with one or both parental species. However, substantial variation in inter-populational crossability and interfertility or karyotype is unlikely in this latter scenario (Schwarzbach & Rieseberg, 2002).

Molecular and crossability/interfertility evidence suggest at least three different origins for *Helianthus anomalus* (Schwarzbach & Rieseberg, 2002). The species has three main chloroplast DNA haplotypes, one apparently derived from *H. annuus* and the other two from *H. petiolaris*. Likewise, the species has three fertility groups that are completely correlated with the distribution of chloroplast DNA haplotypes. Meiotic analyses indicate the fertility groups differ by one or two reciprocal translocations, as would be predicted for multiple origins. However, there was no apparent signature of multiple origins in the nuclear microsatellite data, perhaps due to gene flow among the independently derived lineages subsequent to hybrid speciation.

Patterns of molecular variation in *Helianthus deserticola* were also most consistent with multiple origins (Gross et al., 2003). The species displayed four different chloroplast DNA haplotypes (one from *H. annuus* and three from *H. petiolaris*), polyphyly at microsatellite loci, and considerable inter-populational crossability and interfertility. There was little congruence between the three data sets, however, making it more difficult to rule out the possibility of a single hybrid speciation event followed by introgression with populations of the parental species.

In contrast to *Helianthus anomalus* and *H. deserticola*, *H. paradoxus* is derived from a single hybrid speciation event (Welch & Rieseberg, 2002b). All surveyed populations of the species share a single chloroplast DNA haplotype (derived from *H. annuus*) and form a single clade with 99.8% bootstrap support based on 17 microsatellite loci.

The apparent numbers of origins of the three hybrid species correlates well with the results from the morphological and QTL analyses. Ten of 12 extreme traits in *Helianthus anomalus* and all 10 extreme traits in *H. deserticola* could be re-created by hybridizing populations of the parental species. However, only 11 of 16 extreme traits in *H. paradoxus* could be replicated in synthetic hybrids, implying that the creation of the *H. paradoxus* phenotype may be a rare event. A similar pattern was observed for genetic correlations. Tightly linked QTLs mostly had effects in the same direction with respect to the phenotypes of the apparently multiply derived *H. anomalus* (ϕ = 0.47) and *H. deserticola* (ϕ = 0.53). In contrast, genetic correlations make it difficult to re-create the multi-trait phenotype of *H. paradoxus* (ϕ = 0.32), which appears to have arisen only once.

ECOLOGICAL SELECTION

Theoretical studies indicate that strong ecological selection is a prerequisite for successful hybrid speciation (McCarthy et al., 1995; Buerkle et al., 2000). Also, our studies of phenotypic differentiation have assumed that ecological selection is the underlying force driving divergence. However, it may be that some of the phenotypic differences result from neutral processes or are a byproduct of selection on correlated traits. Correlated selection is likely to be particularly important in hybrid speciation, because the unit of selection is the chromosomal segment rather than individual gene or mutation.

We have employed both comparative and experimental approaches to identify traits likely to have been under selection during hybrid speciation. The comparative approach focuses on traits in the hybrid species that are common to other taxa sharing its habitat. For example, the sand dune endemic, *Helianthus anomalus*, has many traits associated with dune habitats such as very large, cylindrical seeds, rapid early root growth, and succulent leaves (Schwarzbach et al., 2001; Rosenthal et al., 2002). Large seeds are thought to represent an adaptation for burial avoidance, whereas a round or cylindrical shape may prevent seeds from being blown away from the dune habitat. Large seeds also likely contribute to rapid early root growth, which enables seedlings to tap into water reserves deeper in the sand dunes. Finally, succulent leaves may serve to reduce water loss and abrasion by blowing sand (Danin, 1991; Bowers, 1996). *Helianthus deserticola* has many of the classic features of a desert annual, including rapid flowering, reduced height at maturity, and small, narrow leaves (Rosenthal et al., 2002). The first two traits ensure rapid reproduction following heavy seasonal rain, whereas small narrow leaves decrease water loss and avoid fatal overheating (Chapin et al., 1987; Gibson, 1998). *Helianthus paradoxus* shares several traits with other salt-loving or halophytic species, including increased leaf succulence and an efficient mechanism for reducing mineral ion uptake (Welch & Rieseberg, 2002a; Lexer et al., 2003b). Both reduce the toxic effects of sodium and other mineral ions.

Our experimental studies involved the transplantation of reciprocal BC_2 synthetic hybrids into the habitats of the three ancient hybrid species. We then measured the strength of selection on individual traits and (for *Helianthus paradoxus*) the QTLs underlying them. For *H. anomalus*, we focused on leaf ecophysiological traits, phenology, and vegetative biomass, with fitness estimated as reproductive biomass (Ludwig et al., 2004). Although we had predicted that selection in the synthetic hybrids would mostly be in the direction of the *H. anomalus* phenotype, this was not necessarily the case. Only for leaf succulence in the BC_2ann hybrids (backcrosses toward *H. annuus*) and water use efficiency in the BC_2pet hybrids (backcrosses toward *H. petiolaris*) was selection toward the *H. anomalus* phenotype. For most traits, the direction of selection was dependent on the genetic background, suggesting the existence of multiple adaptive peaks for *Helianthus* in the dune habitat. Although these results seem inconsistent with the multiple origins of the species (Schwarzbach & Rieseberg, 2002), it may be that fertility selection or selection on other correlated traits drive early generation hybrids toward just one of these peaks.

Results from the selection analyses in the *Helianthus deserticola* environment were equally difficult to interpret (Gross et al., 2004). For two of the key traits, leaf area and flowering date, selection differentials and gradients differed in sign in both of the BC_2 populations. Thus, while it is clearly feasible for directional selection to produce the *H. deserticola* phenotype for these traits, it is not possible to predict the phenotypic outcome of such selection from the present experiment. For the third key trait, stem diameter, selection in the synthetic hybrids was consistent, but in the opposite direction of the *H. deserticola* phenotype. This result is puzzling and may possibly relate to the experimental design, in which seedlings were propagated at Indiana University and then transported to Utah for transplantation. In such weak and etiolated seedlings, it may be that large stems would be favored, whereas narrow stems might be favored in seedlings propagated under natural, high light conditions.

Only in *Helianthus paradoxus* habitat was the direction of selection in the experimental hybrids consistently in the direction of the *H. paradoxus* phenotype (Lexer et al., 2003b). Positive directional selection was detected for leaf succulence and Ca uptake, two traits that are known to mitigate salt stress in plants. In contrast, the uptake of Na and other toxic mineral ions was strongly negatively selected in the salt marsh habitat. Of greater significance was the observation that QTLs underlying both mineral ion uptake traits and survivorship in the salt marsh had effects in opposing directions (Lexer et al., 2003a). As discussed earlier, this kind of genetic architecture underlies transgressive segregation and provides a simple explanation for how the salt marsh habitat of *H. paradoxus* might have been originally colonized by hybrids.

The QTL data also allowed us to test a fundamental requirement for homoploid hybrid speciation. Homoploid hybrid speciation represents a kind of sympatric or parapatric speciation, and models of speciation with gene flow indicate the strength of selection at individual loci must be greater than the migration rate (Maynard Smith, 1966). Therefore, we calculated selection coefficients for mineral ion QTLs in the salt marsh habitat (Lexer et al., 2003a). The selection coefficients were larger (-0.08 to $+0.13$) than any conceivable migration rate for sunflowers, indicating it would have been feasible for *Helianthus paradoxus* to arise in sympatry or parapatry with its parental species.

CANDIDATE GENES FOR ECOLOGICAL DIVERGENCE

Finally, we have assayed sequence polymorphisms for salt tolerance candidate genes in these same BC_2 populations to determine whether any of the candidate genes map to QTLs of interest, as well as to identify additional genomic regions associated with salt tolerance (Lexer et al., 2004). The salt tolerance candidates were identified from an expressed sequence tag (EST) library for *Helianthus paradoxus* based on homology to genes with known function. One of the 11 genes, a Ca-dependent protein kinase (CDPK), maps coincident with a previously identified QTL for mineral ion uptake. Two additional genes (an ER-type calcium ATPase and a transcriptional regulator) also exhibit a significant fitness effect in the wild. Of course, these studies are correlational only and function will have to be verified by transgenic complementation or RNAi. Nonetheless, they indicate we soon may be able to examine the role of individual genes in ecological divergence and speciation in sunflowers.

CONCLUSIONS AND FUTURE DIRECTIONS

Over the past 15 years, my lab has studied how new hybrid species may arise without a change in chromosome number. Our main contributions include:

(1) Molecular documentation of the hybrid origin of three distinct sunflower species from the same pair of parental species (Rieseberg et al., 1990b; Rieseberg, 1991).

(2) Development of the first genetic map for a homoploid hybrid species (Rieseberg et al., 1993).

(3) Demonstration that homoploid hybrid speciation in wild sunflowers has been accompanied by rapid karyotypic evolution as predicted by verbal models (Rieseberg et al., 1995b; Lai et al., 2005).

(4) Experimental re-creation of homoploid hybrid species in sunflower and demonstration of genomic congruence of synthetic and ancient hybrid species (Rieseberg et al., 1996). This demonstrated the repeatability of homoploid hybrid speciation and the importance of fertility selection in shaping hybrid genomic composition.

(5) Demonstration of the rapid tempo of hybrid speciation based on the large sizes of parental chromosomal blocks in a hybrid sunflower species (Ungerer et al., 1998).

(6) Computer simulation of homoploid hybrid speciation, which verified verbal theory and demonstrated the evolutionary conditions favorable to hybrid origin differ from those required for the independent evolution of the hybrid species (Buerkle et al., 2000).

(7) Re-creation of phenotypes of homoploid hybrid species by experimental hybridization of contemporary parental species populations and transgressive segregation (Lexer et al., 2003b; Rieseberg et al., 2003; Gross et al., 2004; Rosenthal et al., 2005).

(8) Demonstration that complementary gene action is the primary cause of transgressive segregation in experimental sunflower hybrids (Lexer et al., 2003b; Rieseberg et al., 2003).

(9) Discovery that genetic correlations facilitate rather than impede the origin of the hybrid sunflower species (Rieseberg et al., 2003).

(10) Demonstration that the genomic composition of the ancient hybrid sunflower species can be predicted from the QTL analysis of phenotypic traits, implying an important role for ecological selection in shaping hybrid genomic composition (Rieseberg et al., 2003).

(11) Discovery that two of the hybrid sunflower species are multiply derived in nature as predicted from earlier studies of the repeatability of hybrid speciation (Schwarzbach & Rieseberg, 2002; Welch & Rieseberg, 2002b; Gross et al., 2003).

(12) Demonstration that many of the transgressive traits discovered in experimental crosses are under selection in natural hybrid habitats (Lexer et al., 2003a; Ludwig et al., 2004; Gross et al., 2004).

(13) Demonstration that selection coefficients for QTLs underlying ecological divergence in *Helianthus paradoxus* are large enough to account for the origin of the species in parapatry with its parental species (Lexer et al., 2003b).

(14) Identification of several promising candidate genes for ecological divergence in *Helianthus paradoxus* (Lexer et al., 2004).

Of course, there is much left to do. Previously, we attempted to replicate the early stages of hybrid speciation by planting segregating hybrids into the habitat of the ancient hybrid species and identifying traits and QTLs that were under selection. We are currently extending these experiments by asking whether we can detect the molecular signature of selection during hybrid speciation and if the same chromosomal segments that were under selection in the experimental populations were also under selection when the species arose in nature more than 63,000 years ago. Preliminary evidence suggests we can. Microsatellite markers flanking the three most strongly selected QTLs in the *Helianthus paradoxus* habitat had significantly reduced variability as compared with microsatellite loci from unselected regions, yet there was no difference in variability levels between these groups of loci in the parental species (C. Edelist et al., unpublished).

Second, my lab has developed a 3000-gene abiotic stress microarray for sunflower (Lai et al., in press). We wish to ask whether gene expression differences are induced by hybridization and whether these induced differences contribute to ecological divergence and hybrid speciation.

Third, we wish to use the hybrid species system to address the fundamental question of whether adaptive divergence within a species is limited by mutation or by gene flow from the center of each species range (Kirkpatrick & Barton, 1997). That is, why did the parental species not colonize the extreme habitats of the ancient hybrid species? Put another way, was it new hybrid gene combinations or reproductive isolation that allowed the hybrid species to colonize new habitats?

Finally, we will continue our work to identify and characterize the genes underlying ecological divergence in this system. This will involve a combination of EST sequencing (> 70,000 ESTs already sequenced), searches for genes with the signature of positive selection (> 30 identified), candidate gene mapping (close to 300 mapped), microarray analyses, and functional testing using transgenic complementation and RNAi.

Although this review has focused almost exclusively on hybrid sunflower species, there are broader issues that need to be addressed as well. In particular, we need to know how frequently homoploid hybrid speciation occurs in nature, which will require better tools for detecting it in phylogenetic trees. Fortunately, help is on the way. New methods of network reconstruction are under development (reviewed in Linder & Rieseberg, 2004), and the availability of sequence data from numerous nuclear genes should provide far more power for detecting reticulate histories than was possible in the past. This power could be further enhanced if clusters of linked genes were sequenced. Under the assumption of hybrid speciation, separate phylogenetic reconstructions of individual genes that are tightly linked should have the topology of only one side of the hybridization. These reconstructions would be topologically incongruent with reconstructions based upon clusters of genes from the other side of the hybridization.

We also need to know whether many of the discoveries made in the sunflower system can be generalized to other homoploid hybrid species. How are most new hybrid species reproductively isolated from their parental species? Is ecological divergence the rule rather than the exception? Are the habitats of the new hybrid species typically extreme relative to their parental species, or are they more accurately described as intermediate or recombinant? What is the genetic basis of the ecological divergence in hybrids? How repeatable is hybrid speciation in other lineages? Are homoploid hybrid species often multiply derived? What are the characteristics of taxa that make them prone to homoploid hybrid speciation? Hopefully, our sunflower work will provide an experimental and conceptual guide for studies of homoploid hybrid species in other organismal groups.

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